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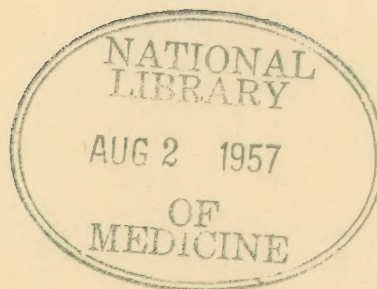
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Army Medical College Epidemiological Research Report

Section 2, Number 17

Research on Cholera Vaccines Treated with Supersonic Waves; Effects of Formalin on Antigenic Properties

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General

The so-called "toxoid immunity," in respect to the immunological effects of anatoxins, has been established by GIENNY, HOPKINS and RANON. In our country numerous contributions toward the preparation of toxoids by treatment with formalin have been made by HIGUCHI, YAOI, KAWASHIMA, ASAKAWA, KUSAMA, HOSOYA, TERAQ, TAKATA, TODA, ISHIMURA, YOHDI and HIDA. Anatoxin antigens have been improved and desired results are being achieved.

According to LESSEPE, Ph., and WERDEAN, A., DOASHIDEN² (TN: * denotes exact transliteration of original Japanese KANA), DUMAS, RANON and KIMURA, it is claimed that in the transformation of Shiga toxin into a toxoid, non-poisonous antigenic properties are not lost when 0.5 per cent or 0.6 per cent formalin is added and incubation at 37 - 39° C is continued for 30 - 90 days.

HOSOYA, KISHINO, TERAQ and TAKATA stated that toxicity was eliminated in pure toxin derived from tetanus toxin by the addition of 0.5 per cent formalin and by maintaining it at blood heat for a five - to nine - day period. No symptoms were indicated when a 5-mg dose (50 times M.L.D.) was injected into the auricular veins of rabbits. The fact that the Shiga toxin can be changed readily into an anatoxin by eliminating the accompanying matter has been proved. In the field of toxins and anatoxins HOSOYA has performed studies on endotoxins and exotoxins.

Because the cholera bacterium toxin is an endotoxin the intracellularly enveloped toxin is freed and absorbed for the first time, and pathological symptoms characteristic of the bacteria are produced when the toxin undergoes proliferation, destruction or autolysis. The nature of this toxin still remains vague, but it is said that the toxic fractions display a protein color reaction, that precipitation is produced by a protein precipitant and that destruction is caused by protein digesting enzymes.

Experiments in which the endotoxins of cholera bacteria have been rendered non-toxic by subjecting the bacteria to the action of supersonic waves have not been reported. It is not difficult, however, to imagine the endotoxins existing in an exposed and diffused state after the bacterial cell has been destroyed. The formation of toxoids by the action of formalin is an extremely interesting study. It was noted in particular that pronounced differences are indicated by virulence tests when comparing antigens treated with supersonic waves to identical antigens not treated in such a manner. First of all, experiments were performed on the virulence and immunizing power of anatoxin antigens treated with supersonic waves. Marked effects were observed by employing formalin in fixed concentrations and by treating for a fixed period.

Chapter I. Test materials.

A. Bacterial strains: The original Kitani strain of cholera bacteria used in previously reported projects was employed. The lethal dose was 0.3 mg for mice. Virulence was maintained by passages through these animals.

B. Laboratory animals: German mice, weighing approximately 12g each, were used for the virulence tests; guinea pigs, weighing

around 200 g were used for the immunization tests. These animals were reared carefully; only the healthy ones were selected for the experiment. Each test group consisted of five animals.

C. Preparation of antigen: Bacteria developing from the Kitani strain, cultured at 37° C for 20 hours in an agar medium (pH 7.4), were suspended in a physiological saline solution at a ratio of 10 mg per cc. Each bacterial suspension was treated for 15 minutes with supersonic waves (600,000 cycles per second). After testing for bacteria-free conditions the resulting supersonic wave-treated antigens were set aside for use in the experiments.

Formalin (0.6 per cent) was added to the supersonic wave-treated antigens. After being shaken thoroughly, the mixtures were placed in an incubator (37° C). These were removed at one-day (24-hour), two-day, three-day, five-day and seven-day intervals and dialyzed with tap water.

The dialyzer was a germ-free intestinal membrane from a cow. Moisture was removed from the outside surfaces after dialysis. The content was sucked into sterile test tubes and specimens which proved to be non-bacterial were preserved.

(I) Control 1 - a supersonic wave-treated antigen (10 mg per cc ratio) which had been treated with supersonic waves for 15 minutes, but which had not been subjected to any other treatment. The purpose of this control was to determine to what extent antigen could be rendered non-toxic by treatment with formalin.

(II) Control 2 - Control 1, to which 0.6 per cent of formalin had been added. It was allowed to stand at room temperature instead of undergoing incubation as in the main experiment. Dialysis was not performed before the tests. The purpose of this control was to determine the nature of its action on animals while in an undialyzed state, following a treatment with formalin.

(III) Control 3 - Control 1 to which 0.4 per cent carbolic acid had been added.

(IV) Control 4 - heated antigen (an antigen produced by adding 0.5 per cent carbolic acid after heating the bacterial suspension at 60° C for one hour). As in the preceding three cases the antigen was employed without any previous testing.

Chapter II. Virulence tests

A. Experimental procedure: The aim of this experiment was to obtain an antigen possessing the highest non-toxic property possible. Experiments were performed to determine the effects of formalin treatment time on the production of a non-poisonous toxoid when incubating an antigen prepared from cholera bacteria by supersonic wave destruction.

Varied doses of each antigen type were injected intraperitoneally into mice during the virulence tests.

B. Results: The mice were kept under observation for a three-day period following the injections. In a case where total death did not occur by the third day the M.L.D. for that particular antigen was established when three out of the five mice died.

The results of the main experiment reveal that total death was produced by 1 mg of antigen which had been treated with formalin for one day, 6 mg when treated for two days, 5 mg when treated for three days, 9 mg when treated for five days and 7 mg when treated for seven days. The controls produced total death with 1 mg (0.8 mg was required with heated antigen).

Table 1. Virulence test on mice

Injection dose		0.1 cc	0.2 cc	0.3 cc	0.4 cc	0.5 cc	0.6 cc	0.7 cc	0.8 cc	0.9 cc	1.0 cc
Bacterial content		1 mg	2 mg	3 mg	4 mg	5 mg	6 mg	7 mg	8 mg	9 mg	10 mg
Furthest transmission time of antigen (7 th d)	1-day	(-)	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$			
	2-day	(-)	(-)	(-)		(-)	(-)	$+\frac{2}{5}$			
	3-day	(-)	(-)	(-)	(-)	(-)	$+\frac{2}{5}$	$+\frac{2}{5}$			
	4-day	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	$+\frac{2}{5}$
	7-day	(-)	(-)	(-)	(-)	(-)	(-)	(-)	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$

Injection dose		0.1 cc	0.2 cc	0.3 cc	0.4 cc	0.5 cc	0.6 cc	0.7 cc	0.8 cc	0.9 cc	1.0 cc
Bacterial content		1 mg	2 mg	3 mg	4 mg	5 mg	6 mg	7 mg	8 mg	9 mg	10 mg
Control 1. Aggravated non-treated vaccine		(-)	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{4}{5}$	$+\frac{4}{5}$	$+\frac{4}{5}$	$+\frac{2}{5}$			
Control 2. Aggravated non-treated vaccine with 0.5 % Formalin		(-)	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{5}{5}$			
Control 3. Aggravated non-treated vaccine with 0.1 % cortisone salt		(-)	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{4}{5}$	$+\frac{2}{5}$			
Control 4. Killed vaccine with 0.5 % cortisone salt		$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$	$+\frac{2}{5}$			

Notes: 1. Immunities represents number of laboratory animals.
 Immunities represents number of surviving animals.

2. Two out of 5 survived with 10 x 0.7 cc killed antigen.
 All survived with 10 x 0.8 cc.

(-) represents $\frac{2}{5} = \frac{2}{5}$

A comparison reveals pronounced deterioration in antigen which had been treated for five days.

2. Control experiments and results:

1. Virulence test on mice using formalin - saline solutions

Formalin was tested for toxicity in order to determine whether or not the addition of formalin in preparing the antigen affects virulence. Formalin was added to a saline solution at the rate 0.6 per cent. The mixture was placed in an incubator and removed at one-day, two-day, three-day, five-day and seven-day intervals. Dilutions were performed in the aforementioned manner. Virulence tests on mice followed outlined procedures.

The results are shown in Table 2. No cases of death due to toxication or to a secondary reaction were observed.

2. The virulence to mice of undestroyed bacterial solution treated with 0.6 per cent formalin

The virulence to mice of antigen treated with superoxide waves and 0.6 per cent formalin already has been described. Assuming that the potent antigenic property is retained, the virulence and antigenic properties of the same amounts of bacterial suspensions were examined by subjecting them to a deactivating process with 0.6 per cent formalin, without subjecting them, however, to a destroying treatment with superoxide waves. In this way the special properties of antigens treated with superoxide waves and 0.6 per cent formalin were investigated.

A suspension was prepared from the killed strain, using a ratio of 10 mg per cc of physiological saline solution. Formalin was added at the rate of 0.6 per cent. The mixture was incubated at 37° C according to a procedure identical to that practiced previously and was removed at one-day, two-day, three-day, five-day and seven-day intervals. Dilutions were performed before testing for germ-free conditions. The antigens were preserved in a refrigerator and later used in the virulence test.

The results are shown in Table 3. The mice survived up to 0.5 mg doses of one-day (formalin) antigen, but died when the dose was increased to 0.6 mg (S.I.D.).

The mice survived 0.3 mg doses of two-day (formalin) antigen, but died when 0.5 mg doses (S.I.D.) were injected.

Death was produced by 0.1 mg doses of three-day, five-day and seven-day (formalin) antigens.

The preceding results are extremely interesting in that the virulence, instead of decreasing appears to have increased after the formalin treatment though what may be interpreted as a satisfactory phenomenon. Because certain bacteria contain antibodies, various factors surrounding toxoid formation through the use of formalin must be borne in mind. In the case where mice were able to survive 5 mg doses of one-day (formalin) antigen, there is the possibility that temporary non-virulence was produced by formalin combining with the toxins which occasionally exist in an adrenergic state. This was observed frequently among the one - and two - day (formalin) antigens.

However, antigens which had been preserved for three-day, five-day and seven-day periods displayed lethal virulence with 2 ug doses.

Table 2. Virulence test on mice using 0.6 per cent formalin + saline solution

Injection dose		0.1 ug	0.2 ug	0.3 ug	0.4 ug	0.5 ug	0.6 ug	0.7 ug
Antigen type	1-day	-	-	-	-	-	-	-
	2-day	-	-	-	-	-	-	-
	3-day	-	-	-	-	-	-	-
	5-day	-	-	-	-	-	-	-
	7-day	-	-	-	-	-	-	-

Table 3. Virulence test with vaccines treated with 0.6 per cent formalin

Injection dose		0.1 ug	0.2 ug	0.3 ug	0.4 ug	0.5 ug	0.6 ug	0.7 ug
Antigen type	1-day	(-)	(-)	(-)	(-)	(-)	$\frac{2}{3}$	$\frac{2}{3}$
	2-day	(-)	(-)	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$
	3-day	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$
	5-day	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$
	7-day	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$

Note: Denominator represents number of laboratory animals. Numerator represents number of surviving animals.

The evaluation of these interesting results constitutes a problem which can be solved only when the true nature of toxins is fully understood. Since this study may be pursued again at another date it may have been premature to conclude that the suspensions containing undestroyed bacteria and treated with 0.6 per cent formalin are wanted for use simply because their virulence proved to be high.

3. Virulence test on mice using antigens treated with increased amounts of formalin

This experiment was performed in order to determine whether varying amounts of formalin would affect the virulence of the antigen and whether amounts over 0.6 per cent would annihilate the speed with which non-toxicity is achieved during the toxic-formulation period.

Formalin was added to antigens (identical to those above) at a rate of six per cent (or 12 times the previous volume). The experiment followed the same procedure. Intraperitoneal injections were performed as above.

The mice survived 0.2 mg doses of one-day (formalin) antigen, but died when 0.3 mg doses (U.L.D.) were injected. The U.L.D. for two-day (formalin) antigen was 0.3 mg. The mice survived 0.4 mg doses of three-day, five-day and seven-day (formalin) antigens but died when 0.5 mg doses (U.L.D.) were introduced. These results reveal that although toxicity is feared from the third day of immersion, no particularly favorable effects can be produced by using formalin in excessive amounts.

Chapter III. Immunization tests

A. Experimental procedure:

Healthy mice weighing about 200 g each were selected for the tests after being raised for a five-day period following their purchase. Each group consisted of five mice.

1. Immunization method:

In the first series, each type of antigen was injected intraperitoneally in a dose equivalent to the minimum lethal dose for mice, after removing the hair from the lower abdominal region of the animal and after thoroughly sterilizing that region.

Twice the original dose was injected in the same manner during the second series, seven days after the first series.

Simultaneous injections of live bacteria which had been diluted in a physiological saline solution were made 12 days after the second series.

Control antigens containing the same amount of bacteria were classified in the manner shown below.

2. Immunization injection doses:

Antigens which had been subjected to dialysis and treatment with superoxide waves and formalin were employed in doses of 0.1 mg (one-day formalin treatment; dialyzed), 0.3 mg (two-day formalin treatment; dialyzed), 0.5 mg (three-day formalin treatment; dialyzed), 0.9 mg (five-day formalin treatment; dialyzed) and 0.7 mg (seven-day formalin treatment; dialyzed).

Doses were 0.1 mg for Controls 1, 2, and 3 and 0.5 mg for Control 4.

Live bacteria used in the immunization tests were from the Miami strain, having an U.L.D. of 0.3 mg. Suspensions were prepared by adding 6 mg of bacteria obtained from an 18-hour culture to 1 cc of physiological saline solution. The 20-unit suspension contained 12 mg per cc of physiological saline solution; the 30-unit suspension contained 18 mg per cc. Each was injected in 0.5-cc doses.

3. Observation methods:

During the tests, the animals were kept in the same room and reared with extreme care. Expected nutrition, temperatures and other suitable conditions which might have contributed to their death were attended. Observations were made several times a day; supervision was rigid.

A. Results:

The results obtained from experiments on immunization with antigens treated with 0.5 per cent formalin and superoxide waves are presented below.

1. Three out of five animals survived 10 units of one-day (formalin) antigen. The same number survived injections of 20 and 30 units. The mean value was approximately 70 per cent. The reason for this was the short formalin-treatment time. (See Tables 6, 7 and 8.)

2. Two out of five animals survived 10-unit injections of two-day (formalin) antigen. Three survived 20-unit injections; all survived 30-unit injections. The mean value was 65 per cent, not differing in any great degree from results produced by the preceding antigen.

3. Although a considerable decrease in virulence occurred when 0.5 per cent of three-day (formalin) antigen was used in the virulence tests, the same antigen type produced a high number of deaths during immunization tests. Because a considerable number died after the second series of immunization injections, it was believed necessary to consider the effects of a formalin-oxide solution on this, too, as already proved by the results in Table 2, such effects were not present.

4. Because of the virulence of 0.7 ug, the five-day (formalin) antigen appeared to be the most satisfactory in the experiment. Four out of five animals survived 10-, 20- and 30-unit injections employed in the immunization tests. The mean value was 80 per cent.

5. Five out of five animals survived 10-, 20- and 30-unit injections of seven-day (formalin) antigen, producing a mean value of 100 per cent. The manifestation of antigenic strength was complete and toxic formation was adequate.

The A.L.D. for mice was 0.7 ug; antigenic strength was observed to be in direct proportion to this value.

The fact that antibodies can be prepared from atypical bacteria after a 30-day treatment already has been mentioned in experimental reports. On this basis it became possible to observe the so-called "toxic transition" of a culture toxin which had been prepared by treating the bacteria with superoxide waves and 0.5 per cent formalin.

A summary of the foregoing results of virulence tests is given in Table 8.

The mean values of the immunization tests, as shown in Table 10 (VII: Summary), are 57 per cent for one-day (formalin) antigen, 53 per cent for two-day (formalin) antigen, 90 per cent for

three-day (formalin) antigen, 80 per cent for five-day (formalin) antigen and 100 per cent for seven-day (formalin) antigen. Virulence was weak; immunization power was low even at five days. Controls 1 and 2 indicated a value of 85 per cent; Control 3 indicated 90 per cent.

Summary and Conclusion

1. The preparation of bacterium possessing high antigenic strength and low toxicity was possible by treating with formalin a suspension of cholera bacteria whose cells had been destroyed by supersonic waves.

2. A formalin concentration of 0.6 per cent proved to be adequate for this purpose. Higher concentrations were unnecessary.

3. Allowing the suspension to stand at "blood heat" for a seven-day period after adding formalin proved to be ideal.

4. No pronounced weakening of virulence was observed by treating with formalin a bacterial suspension not previously subjected to the action of supersonic waves.

5. Immunization injection doses can be determined from their respective toxicity values. Their immunization strengths follow the order shown below.

(a) Supersonic wave-treated cholera bacteria; 0.6 per cent formalin; allowed to stand at blood heat for seven days; dialyzed.

(b) Supersonic wave-treated cholera bacteria; 0.4 per cent corticoid salt.

(c) Supersonic wave-treated cholera bacteria; no formalin treatment.

(d) Supersonic wave-treated cholera bacteria; 0.6 per cent formalin; allowed to stand at room temperature; not dialyzed.

(e) Cholera bacteria suspension; heated for one hour at 60° C.

Table 4. Virulence test on mice using vaccines treated with aqueous waves and 0.6 per cent formalin

Infection dose		0.1 ml	0.2 ml	0.3 ml	0.4 ml	0.5 ml	0.6 ml	0.7 ml
Antigen type	1-day	(-)	(-)	$\frac{2}{3}$	$\frac{0}{3}$	$\frac{0}{3}$	$\frac{2}{3}$	$\frac{0}{3}$
	2-day	(-)	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{0}{3}$	$\frac{2}{3}$	$\frac{0}{3}$
	3-day	(-)	(-)	(-)	(-)	$\frac{12}{13}$	$\frac{12}{13}$	$\frac{12}{13}$
	4-day	(-)	(-)	(-)	(-)	$\frac{12}{13}$	$\frac{2}{3}$	$\frac{12}{13}$
	7-day	(-)	(-)	(-)	(-)	$\frac{12}{13}$	$\frac{12}{13}$	$\frac{12}{13}$

Table 5. Immunization test (marmots; 10 units = 3 mg)

Immunity and live bacteria injection; progress	No.	Immunization unit volume	Weight (g)	Markings	First series 16 June		Second series 23 June		Live bacteria injection 5 July Date of death	Elapsed time (days)					Number surviving
					Injection dose (mg)	Date of death	Injection dose (mg)	Date of death		1	2	3	4	5	
1-day	1	1 mg	200	red	1		2		6 Jul	+	-	-	-	-	$\frac{3}{5}$
	2		200	blue	1		2			-	-	-	-	-	
	3		210	purple	1		2			-	-	-	-	-	
	4		200	yellow	1		2		7 Jul	-	-	-	-	-	
	5		200	-	1		2			-	+	-	-	-	
2-day	6	6 mg	210	red	6		12		7 Jul	+	-	-	-	-	$\frac{2}{5}$
	7		200	blue	6		12			-	-	-	-	-	
	8		210	purple	6		12		6 Jul	+	-	-	-	-	
	9		210	yellow	6		12		6 Jul	+	-	-	-	-	
	10		210	-	6		12			-	-	-	-	-	
3-day	11	5 mg	210	red	5		10			-	-	-	-	-	$4\frac{1}{4}$
	12		200	blue	5		10			-	-	-	-	-	
	13		210	purple	5		10			-	-	-	-	-	
	14		210	yellow	5	18 Jun	10			-	-	-	-	-	
	15		210	-	5		10			-	-	-	-	-	
5-day	16	9 mg	200	red	9		18		7 Jul	+	-	-	-	-	$4\frac{1}{5}$
	17		200	blue	9		18			-	-	-	-	-	
	18		210	purple	9		18			-	-	-	-	-	
	19		200	yellow	9		18			-	-	-	-	-	
	20		200	-	9		18			-	-	-	-	-	
7-day	21	7 mg	200	red	7		14			-	-	-	-	-	$\frac{5}{5}$
	22		200	blue	7		14			-	-	-	-	-	
	23		210	purple	7		14			-	-	-	-	-	
	24		200	yellow	7		14			-	-	-	-	-	
	25		200	-	7		14			-	-	-	-	-	
S.S.V. K I	26	1 mg	200	red	1		2		6 Jul	+	-	-	-	-	$4\frac{1}{5}$
	27		200	blue	1		2			-	-	-	-	-	
	28		200	purple	1		2			-	-	-	-	-	
	29		200	yellow	1		2			-	-	-	-	-	
	30		200	-	1		2			-	-	-	-	-	
K II 0.6 % formalin (not dialyzed)	31	1 mg	200	red	1		2			-	-	-	-	-	$\frac{5}{5}$
	32		200	blue	1		2			-	-	-	-	-	
	33		200	purple	1		2			-	-	-	-	-	
	34		200	yellow	1		2			-	-	-	-	-	
	35		190	-	1		2			-	-	-	-	-	
K III 0.4 % carbolic acid	36	1 mg	200	red	1		2			-	-	-	-	-	$4\frac{1}{5}$
	37		200	blue	1		2			-	-	-	-	-	
	38		200	purple	1		2			-	-	-	-	-	
	39		200	yellow	1		2		6 Jul	-	-	-	-	-	
	40		200	-	1		2			-	-	-	-	-	
0.5 % carbolic acid. Heated	41	0.5 mg	200	red	0.5		1.6			-	-	-	-	-	5/5
	42		210	blue	0.5		1.6			-	-	-	-	-	
	43		210	purple	0.5		1.6			-	-	-	-	-	
	44		210	yellow	0.5		1.6			-	-	-	-	-	
	45		210	-	0.5		1.6			-	-	-	-	-	

Table 6. Immunization test (marmosets; 20 units = 6 mg)

Immunity and live bacteria injection; progress	No.	Immunization unit volume	Weight (g)	Markings	First series		Second series		Live bacteria in- jection 23 August dose (mg)	Date of death	Elapsed time (days)					Number surviving
					Injection dose (mg)	Date of death	Injection dose (mg)	Date of death			1	2	3	4	5	
1-day	1	1 mg	200	red	1		2		6	28 Aug	-	-	-	-	-	4-5
	2		220	blue	1		2		6		-	-	-	-	-	
	3		210	purple	1		2		6		-	-	-	-	-	
	4		220	yellow	1		2		6		-	-	-	-	-	
	5		230	-	1		2		6		-	-	-	-	-	
2-day	6	6 mg	230	red	6		12		6	28 Aug 24 Aug	-	-	-	-	-	3-5
	7		200	blue	6		12		6		-	-	-	-	-	
	8		230	purple	6		12		6		-	-	-	-	-	
	9		200	yellow	6		12		6		-	-	-	-	-	
	10		230	-	6		12		6		-	-	-	-	-	
3-day	11	5 mg	230	red	5		10	13 Aug	6		-	-	-	-	-	3-5
	12		230	blue	5		10	12 Aug	6		-	-	-	-	-	
	13		230	purple	5		10		6		-	-	-	-	-	
	14		240	yellow	5		10		6		-	-	-	-	-	
	15		220	-	5		10		6		-	-	-	-	-	
5-day	16	9 mg	200	red	9		18		6	24 Aug	-	-	-	-	-	4-5
	17		230	blue	9		18		6		-	-	-	-	-	
	18		230	purple	9		18		6		-	-	-	-	-	
	19		200	yellow	9		18		6		-	-	-	-	-	
	20		230	-	9		18		6		-	-	-	-	-	
7-day	21	7 mg	200	red	7		14		6		-	-	-	-	-	5-5
	22		230	blue	7		14		6		-	-	-	-	-	
	23		230	purple	7		14		6		-	-	-	-	-	
	24		230	yellow	7		14		6		-	-	-	-	-	
	25		200	-	7		14		6		-	-	-	-	-	
U.S.V. K I	26	1 mg	220	red	1		2		6		-	-	-	-	-	5-5
	27		200	blue	1		2		6		-	-	-	-	-	
	28		220	purple	1		2		6		-	-	-	-	-	
	29		240	yellow	1		2		6		-	-	-	-	-	
	30		220	-	1		2		6		-	-	-	-	-	
K II 0.6 % formalin (not dialyzed)	31	1 mg	200	red	1		2		6		-	-	-	-	-	5-5
	32		220	blue	1		2		6		-	-	-	-	-	
	33		200	purple	1		2		6		-	-	-	-	-	
	34		220	yellow	1		2		6		-	-	-	-	-	
	35		220	-	1		2		6		-	-	-	-	-	
K III 0.4 % carbolic acid	36	1 mg	230	red	1		2		6		-	-	-	-	-	5-5
	37		200	blue	1		2		6		-	-	-	-	-	
	38		220	purple	1		2		6		-	-	-	-	-	
	39		200	yellow	1		2		6		-	-	-	-	-	
	40		300	-	1		2		6		-	-	-	-	-	
0.5 % carbolic acid. Heated	41	0.8 mg	220	red	0.8		1.6		6	25 Aug	-	-	-	-	-	4-5
	42		220	blue	0.8		1.6		6		-	-	-	-	-	
	43		220	purple	0.8		1.6		6		-	-	-	-	-	
	44		230	yellow	0.8		1.6		6		-	-	-	-	-	
	45		220	-	0.8		1.6		6		-	-	-	-	-	

Table 7. Immunization test (marmosets; 30 units = 9 mg)

Immunity and live bacteria injection; J-group	No.	Immunization unit volume	Weight (g)	Markings	First series 19 August		Second series - August		Live bacteria injection 1 September		Elapsed time (days)					Number surviving
					Injection dose (mg)	Date of death	Injection dose (mg)	Date of death	Injection dose (mg)	Date of death	1	2	3	4	5	
1-day	1	1 mg	220	red	1		2		9		-	-	-	-	-	$\frac{4}{5}$
	2		200	blue	1		2		9		-	-	-	-	-	
	3		220	purple	1		2		9		-	-	-	-	-	
	4		210	yellow	1		2		9	2 Sep	+	-	-	-	-	
	5		200	-	1		2		9		-	-	-	-	-	
2-day	6	6 mg	220	red	6		12		9	4 Sep	-	-	+	-	-	$\frac{3}{5}$
	7		250	blue	6		12		9		-	-	-	-	-	
	8		220	purple	6		12		9		-	-	-	-	-	
	9		220	yellow	6		12		9	4 Sep	-	-	-	-	-	
	10		210	-	6		12		9		-	-	+	-	-	
3-day	11	5 mg	220	red	5		10		9	2 Sep	+	-	-	-	-	$\frac{4}{5}$
	12		230	blue	5		10		9		-	-	-	-	-	
	13		240	purple	5		10		9		-	-	-	-	-	
	14		220	yellow	5		10		9		-	-	-	-	-	
	15		210	-	5		10		9		-	-	-	-	-	
5-day	16	9 mg	230	red	9		18		9	3 Sep	+	+	-	-	-	$\frac{4}{5}$
	17		220	blue	9		18		9		-	-	-	-	-	
	18		200	purple	9		18		9		-	-	-	-	-	
	19		200	yellow	9		18		9		-	-	-	-	-	
	20		200	-	9		18		9		-	-	-	-	-	
7-day	21	7 mg	220	red	7		14		9		-	-	-	-	-	$\frac{5}{5}$
	22		230	blue	7		14		9		-	-	-	-	-	
	23		220	purple	7		14		9		-	-	-	-	-	
	24		200	yellow	7		14		9		-	-	-	-	-	
	25		200	-	7		14		9		-	-	-	-	-	
U.S.V. K I	26	1 mg	210	red	1		2		9	3 Sep	-	-	-	-	-	$\frac{4}{5}$
	27		230	blue	1		2		9		-	-	-	-	-	
	28		210	purple	1		2		9		-	-	-	-	-	
	29		200	yellow	1		2		9		-	-	-	-	-	
	30		200	-	1		2		9		-	-	-	-	-	
K II 0.6 % formalin (not dialysed)	31	1 mg	220	red	1		2		9	2 Sep	+	+	-	-	-	$\frac{3}{5}$
	32		230	blue	1		2		9	2 Sep	+	+	-	-	-	
	33		200	purple	1		2		9		-	-	-	-	-	
	34		210	yellow	1		2		9		-	-	-	-	-	
	35		240	-	1		2		9		-	-	-	-	-	
K III 0.4 % carbolic acid	36	1 mg	230	red	1		2		9		-	-	-	-	-	$\frac{4}{4}$
	37		210	blue	1		2		9		-	-	-	-	-	
	38		230	purple	1		2		9		-	-	-	-	-	
	39		200	yellow	1		2		9		-	-	-	-	-	
	40		200	-	1		2	23 Aug	9		-	-	-	-	-	
0.5 % carbolic acid. Sealed	41	0.8 mg	230	red	0.8		1.6	23 Aug	9	2 Sep	+	-	-	-	-	$\frac{3}{4}$
	42		220	blue	0.8		1.6		9		-	-	-	-	-	
	43		230	purple	0.8		1.6		9	3 Sep	-	-	-	-	-	
	44		230	yellow	0.8		1.6		9	2 Sep	+	-	-	-	-	
	45		220	-	0.8		1.6		9	2 Sep	+	-	-	-	-	

Antigen type

Table 8. Virulence and immunisation test results

Antigen type	Treated with 0.6 % formalin dialysed						K1	K2	K3	K4
	1-day	2-day	3-day	5-day	7-day					
Virulence test	1 mg	6 mg	5 mg	9 mg	7 mg		1 mg	1 mg	1 mg	0.8 mg
10 units	3/5	2/5	4/4	4/5	5/5		4/5	5/5	4/5	5/5
20 units	4/5	3/5	3/3	4/5	5/5		5/5	5/5	5/5	4/5
30 units	4/5	3/5	4/5	4/5	5/5		4/5	3/5	4/5	1/5
Total	11/15	8/5	11/12	12/15	15/15		13/15	13/15	13/14	10/14
Rate of survival (%)	73	53	92	80	100		87	87	93	71

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